VERIFICATION OF TRANSLATION

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declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

2. That the attached document prepared by me is, to the best of my knowledge and belief, a true and correct translation of a certified copy of the following application.

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   Entitled: "METHOD OF MEASURING INSULIN RECEPTOR α SUBUNIT"
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APPENDIX 2

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【Name of Document】 Specification 1

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[Document Name] Specification

[Title of the Invention] METHOD FOR DETERMINATION OF INSULIN RECEPTOR ALPHA SUBUNIT

[Claims]

[Claim 1] A method for measuring a free insulin receptor α-subunit in blood, wherein the method comprises the steps of:

(1) contacting a blood sample with an antibody recognizing the insulin receptor α-subunit;

(2) detecting binding of said antibody to the insulin receptor α-subunit present in blood; and

(3) determining the amount of free insulin receptor α-subunit in blood based on the level of binding detected between said antibody and subunit.

[Claim 2] The method of claim 1, wherein the antibody recognizing the insulin receptor α-subunit is a first antibody that is bound to a solid phase or comprises a label that can be bound to a solid phase, and the method comprises the step of detecting the insulin receptor α-subunit bound to the first antibody by binding a second antibody recognizing the insulin receptor α-subunit.

[Claim 3] A reagent for measuring a free insulin receptor α-subunit in blood, wherein the reagent comprises an antibody recognizing the insulin receptor α-subunit.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to methods for measuring a free insulin receptor α-subunit in blood.

[0002]

[Prior Art]

Insulin is a hormone that plays an important role in the metabolic regulation of glucose, an energy source for living organisms. Produced in pancreatic Langerhans β cells, insulin acts on cells carrying insulin receptors and promotes the uptake of glucose by these cells. The blood-sugar level in the body is maintained within an appropriate range by the function of insulin. Diabetes is one of the pathological conditions caused by insufficient insulin function due to some cause.

[0003]

Major causes of insufficient insulin function include abnormal insulin secretion and decreased sensitivity to insulin. The former is called type 1 diabetes mellitus. Since responsiveness to insulin is maintained in type 1 diabetes mellitus, blood sugar level can be

controlled by administering insulin. Type 1 diabetes mellitus is also called insulin-dependent diabetes mellitus (IDDM), and is the main cause of juvenile diabetes.

[0004]

On the other hand, the latter is called type 2 diabetes mellitus. Type 2 diabetes mellitus is also called non-insulin dependent diabetes mellitus (NIDDM), and is the type of diabetes frequently found in adults. In Japan, 95% of diabetes patients are said to have type 2 diabetes mellitus. Since the body's responsiveness to insulin is decreased in these patients, even an insulin administration cannot regulate the blood sugar level. Type 2 diabetes mellitus is thought to develop due to several genetic defects and environmental factors such as obesity, stress, and aging. At present, approximately 7,000,000 type 2 diabetes patients are said to exist in Japan, and the number is increasing with the aging of the population. The number of patients is even predicted to be as many as 14,000,000, when including prediabetes patients. Therefore, the diagnosis and treatment of type 2 diabetes mellitus is an important research issue for the modern society.

[0005]

To date, the causative gene of type 2 diabetes mellitus has not been revealed. Presumed candidate genes are genes of factors involved in the mechanism of insulin action, or genes of factors involved in insulin secretion. Factors thought to be involved in insulin action are:

insulin receptor,
insulin receptor substrate-1 (IRS-1),
glucose transporter type 4, etc.

Genes of factors predicted to be involved in insulin secretion are:

glucose transporter type 2,
glucokinase,
mitochondrial genes, etc.

[0006]

For insulin to act on a target cell, it must bind to the insulin receptor present on the target cell membrane. Furthermore, there are many reports of insulin resistance in the early stage of type 2 diabetes (Document 1/Taylor, S. I. Diabetes 41: 1473-1490, 1992). In view of these facts, the relationship between insulin receptor abnormalities and diabetes has also been examined. If abnormalities are present in insulin receptor function, strong insulin resistance will arise, resulting in severe diabetes.

[0007]

Recently, many insulin receptor abnormalities have been discovered by researchers including the present inventors, and it is becoming evident that test results and symptoms of

patients vary depending on the type of mutation (Document 2/ M. Taira *et al.*, Science 245: 63-66, 1989; Document 3/ F. Shimada *et al.*, Lancet. 335: 1179-1181, 1990). This suggests that a part of the pathogenesis of type 2 diabetes mellitus may be defects in the insulin receptor gene. The present inventors have actually identified one of the polymorphisms that allow genetic diagnosis of type 2 diabetes mellitus, and have already filed a patent application (Patent Document 1/ Unexamined Published Japanese Patent Application No. (JP-A) H08-103280).

[0008]

Insulin receptors are heterotetrameric receptor proteins composed of two subunits, α and β. The α-subunit is present outside the cell and the β-subunit penetrates the cell membrane. The α-subunit is linked to the extracellular domain of the β-subunit via an SS bond through an SH group in a cysteine residue on the C-terminal side thereof.

When insulin binds to the α-subunit, a tyrosine residue in the intracellular domain of the β-subunit is autophosphorylated, and the insulin signal is transmitted to the cell. After binding with insulin, the insulin receptor present in the cell membrane is then taken into the cell by endocytosis (the half-life of the receptor is seven hours). The number of insulin receptors decrease with the increase of insulin concentration. This is called down regulation.

[0009]

In the polymorphism of insulin receptor found by the present inventors, Thr at position 831 in the β-subunit is mutated to Ala (IRA831). Insulin receptor dysfunction caused by this amino acid substitution has not been confirmed. However, genetic statistics indicated that there is a strong relation between IRA831 and type 2 diabetes mellitus.

Disorders due to receptor abnormalities or the presence of free receptors in blood have recently been reported for some diseases (Document 4/Frode, T.S., Tenconi, P., Debiasi, M.R., Medeiros, Y.S., "Tumour necrosis factor-alpha, interleukin-2 soluble receptor and different inflammatory parameters in patients with rheumatoid arthritis." Mediators Inflamm. 2002 Dec; 11 (6): 345-9; Document 5/Baron, A.T., Cora, E.M., Lafky, J.M., Boardman, C.H., Buenafe, M.C., Rademaker, A., Liu, D., Fishman, D.A., Podratz, K.C., Maihle, N.J., "Soluble Epidermal Growth Factor Receptor (sEGFR/sErbB1) as a potential Risk, Screening, and Diagnostic Serum Biomarker of Epithelial Ovarian Cancer." Cancer Epidemiol Biomarkers Prev., 2003 Feb; 12 (2): 103-13; Document 6/Beguin, Y. "Soluble transferrin receptor for the evaluation of erythropoiesis and iron status." Clin. Chem. Acta., 2003 Mar; 329 (1-2): 9-22). Furthermore, hyperglycemia and hyperinsulinemia have been observed in transgenic mice that release the insulin receptor α-subunit into the blood (Document 7/ ERIK M. SCHAEFER *et al.* DIABETES vol. 43, 143-153; 1994). However, in humans, the presence of free insulin receptors in blood has not been reported.

[0010]

[Document 1] Taylor,S.I. Diabetes 41: 1473-1490,1992
[Document 2] M. Taira et al., Science 245: 63-66,1989
[Document 3] F.Shimada et al., Lancet335: 1179-1181,1990
[Document 4] Frode TS et al., Mediators Inflamm. 2002 Dec; 11 (6): 345-9
[Document 5] Baron AT et al., Cancer Epidemiol Biomarkers Prev, 2003 Feb; 12 (2): 103-13
[Document 6] Beguin Y, Clin Chem Acta, 2003 Mar; 329 (1-2): 9-22
[Document 7] ERIK M. SCHAEFER et al. DIABETES vol.43, 143-153; 1994
[Patent Document 1] Unexamined Published Japanese Patent Application No. (JP-A) H08-103280

[0011]

[Problems to be Solved by the Invention]

An objective of the present invention is to provide methods for measuring free insulin receptor α-subunit present in blood.

[0012]

[Means for Solving the Problems]

The present inventors continued to study causes hindering insulin function. As a result, the insulin receptor α-subunit existing in the blood in a free form was revealed to hinder insulin action and cause hyperglycemia. The present inventors thought it necessary to establish a system for measuring the insulin receptor α-subunit in order to advance analyses on the relationship between diabetes and free insulin receptor α-subunit in the living body. Consequently, the present inventors established methods for measuring the free α-subunit in blood, and completed the invention. More specifically, the present invention relates to the following methods for measuring the insulin receptor α-subunit and reagents for the measurement.

[1] A method for measuring a free insulin receptor α-subunit in blood, wherein the method comprises the steps of:
(1) contacting a blood sample with an antibody recognizing the insulin receptor α-subunit;
(2) detecting binding of said antibody to the insulin receptor α-subunit present in blood; and
(3) determining the amount of free insulin receptor α-subunit in blood based on the level of binding detected between said antibody and subunit.

[2] The method of [1], wherein the antibody recognizing the insulin receptor α-subunit is a first antibody that is bound to a solid phase or comprises a label that can be bound to a solid phase, and the method comprises the step of detecting the insulin receptor α-subunit bound to the first antibody by binding a second antibody recognizing the insulin receptor α-subunit.

[3] A reagent for measuring a free insulin receptor α-subunit in blood, wherein the

reagent comprises an antibody recognizing the insulin receptor α-subunit.

[0013]

[Mode for Carrying Out the Invention]

The present invention provides methods for measuring free insulin receptor α-subunits in blood comprising the following steps:

(1) contacting a blood sample with an antibody recognizing the insulin receptor α-subunit;

(2) detecting binding of said antibody to the insulin receptor α-subunit present in blood; and

(3) determining the amount of free insulin receptor α-subunit in blood based on the level of binding detected between said antibody and subunit.

[0014]

In the present invention, the term "free" means that the molecule is dispersed in blood. Insulin receptors are proteins typically localized on cell membrane surfaces. Furthermore, it has been shown that insulin receptors are highly expressed in skeletal muscles, adipose tissues, liver, brain, and such. In other words, marked expression of the insulin receptor is not observed in blood cells such as lymphocytes. Therefore, it was unknown whether a free insulin receptor exists in blood. It was the present inventors who revealed that a free insulin receptor subunit is indeed present in blood.

[0015]

A method for measuring the insulin receptor was known (Human insulin receptor radioimmunoassay: applicablity to insulin-resistant state. Am. J. Physiol. 257 (Endocrinol. Metab. 20) E451-E457, 1989). However, the existence of free insulin receptor α-subunit in blood was not known, and methods for measuring were not established. There is only one document indicating the possibility of the presence of the α-subunit in blood (J. Clin. Endocrinol. Metab. 1992 May; 74 (5): 1116-21).

[0016]

In the present invention, the insulin receptor α-subunit present in blood can be measured by its binding to an antibody that recognizes the insulin receptor α-subunit. Antibodies used in the present invention that recognize the insulin receptor α-subunit can be obtained by known methods.

[0017]

Antibodies necessary for the present invention can be obtained, for example, by using recombinant insulin receptors as an immunogen. The present inventors demonstrated that the insulin receptor α-subunit could be secreted outside of the cell by using cDNAs encoding amino acid sequences as described below. Secretory polypeptides that can be obtained in this manner, fragments thereof, and such may be utilized as immunogens for obtaining the antibodies of the present invention. These polypeptides can be obtained by transforming suitable hosts with

vectors that harbor cDNA of a known insulin receptor α-subunit or the nucleotide sequence of SEQ ID NO: 1 (or fragments thereof) in an expressible manner.

[0018]

DNA comprising the necessary nucleotide sequence can be cloned using mRNA prepared from tissues where the insulin receptor α-subunit is expressed. Alternatively, DNA comprising the nucleotide sequence shown in SEQ ID NO: 1 can be obtained by modifying a known nucleotide sequence of the cDNA of an insulin receptor α-subunit. Recombinant insulin receptor α-subunits expressed in this manner are preferred as immunogens for obtaining antibodies to be used in this invention.

[0019]

Alternatively, domain peptides of the insulin receptor α-subunit can be used as an immunogen. Domain peptides used as an immunogen can be easily synthesized using a peptide synthesizer. Synthetic peptides can be prepared as immunogens by linking them to carrier proteins.

The maleimidobenzoyl-*N*-hydrosuccinimide method (hereinafter abbreviated as the MBS method) and such are generally used to link synthetic peptides to carrier proteins. Specifically, a cysteine is introduced into a synthetic peptide, and the peptide is cross-linked to KLH by the MBS method through the cysteine's SH group. The cysteine residue may be introduced at the N terminus or C terminus of the synthesized peptide. As carrier proteins, a suitable protein other than KLH, such as bovine serum albumin may be used. KLH is one of the preferred carrier proteins because of its high immunogenicity.

[0020]

Immunogens obtained in this manner are mixed with suitable adjuvants, and used to immunize animals. Known adjuvants include Freund's complete adjuvant (FCA) and incomplete adjuvant. The immunization is repeated with appropriate intervals until an increase in antibody titer is confirmed. There are no particular limitations on the animals to be immunized in the present invention. Specifically, mice, rats, rabbits, or such animals commonly used for immunization may be used.

When obtaining the antibodies as monoclonal antibodies, animals that are advantageous for producing them may be used. For example, in mice, many myeloma cell lines for cell fusion are known, and techniques capable of establishing hybridomas with a high probability have already been developed. Therefore, mice are one of the preferable animals for immunization.

Furthermore, immune treatment is not limited to *in vivo* treatment. Methods for immunologically sensitizing cultured immunocompetent cells *in vitro* can also be employed. Antibody-producing cells obtained by these methods are transformed and cloned. The method

for transforming antibody-producing cells to obtain monoclonal antibodies is not limited to cell fusion. For example, methods for obtaining transformants that can be cloned via virus infection are known.

[0021]

Hybridomas that produce monoclonal antibodies to be used for the present invention can be screened based on their reactivities to various antigens. Specifically, antibody-producing cells are first selected by using, as an indicator, their binding activities toward the insulin receptor α-subunit or its domain peptides that were used as antigens. Positive clones selected by this screening are subcloned as necessary.

[0022]

After culturing the established hybridomas under suitable conditions, produced antibodies are collected to yield monoclonal antibodies to be used in the present invention. When the hybridomas are homohybridomas, they can be cultured *in vivo* by inoculating them intraperitoneally to syngeneic animals. In this case, monoclonal antibodies are collected as peritoneal fluid. When heterohybridomas are used, they can be cultured *in vivo* using nude mice as a host.

[0023]

In vivo cultures, as well as cultures outside the body in appropriate culture environments are generally conducted. For example, basal media such as RPMI 1640 and DMEM are generally used as the media for hybridomas. Animal serum and such additives can be added to these media to maintain the antibody producing ability at a high level. When hybridomas are cultured outside of the body, monoclonal antibodies can be collected as a culture supernatant. The culture supernatant can be collected by separating it from cells after culturing, or, when using a culturing apparatus that applies hollow fibers, it can be continuously collected while culturing.

[0024]

Monoclonal antibodies collected as peritoneal fluid or culture supernatants are prepared into monoclonal antibodies used in the present invention by separating the immunoglobulin fraction by saturated ammonium sulfate precipitation followed by purification steps including gel filtration and ion exchange chromatography. In addition, if the monoclonal antibodies are IgGs, purification methods based on affinity chromatography with a protein A or protein G column are effective.

[0025]

On the other hand, to obtain the antibodies used in the present invention as polyclonal antibodies, blood is drawn from individuals whose antibody titer has increased after immunization, and the sera are separated to obtain anti-sera. Immunoglobulins are purified

from anti-sera by known methods to prepare the antibodies to be used in the present invention. If immunoaffinity chromatography using insulin receptor α-subunit as a ligand is used in combination with immunoglobulin purification, insulin receptor α-subunit-specific antibodies can be obtained.

[0026]

When antibodies against the insulin receptor α-subunit contact the insulin receptor α-subunit, the antibodies bind to the antigenic determinants (epitopes) that the antibodies recognize thorough the antigen-antibody reaction. The binding of antibodies to antigens can be detected by various immunoassay principles. Immunoassays can be broadly categorized into heterogeneous analysis methods and homogeneous analysis methods. To keep the sensitivities and specificities of immunoassays at a high level, monoclonal antibodies are preferably used. The present invention's methods for measuring the insulin receptor α-subunit using a variety of immunoassay formats will be described in detail.

[0027]

First, the method for measuring the insulin receptor α-subunit using heterogeneous immunoassays will be described. In heterogeneous immunoassays, mechanisms for separately detecting insulin receptor α-subunit-bound and -unbound antibodies are required.

[0028]

To facilitate the separation, immobilized reagents are generally used. For example, first, a solid phase onto which antibodies recognizing insulin receptor α-subunit has been immobilized is prepared (immobilized antibody). The insulin receptor α-subunit is bound to this, and then reacted with a labeled second antibody.

[0029]

When the solid phase is separated from the liquid phase and then washed as necessary, an amount of second antibody proportional to the concentration of insulin receptor α-subunit remains on the solid phase. If the second antibody is labeled, the insulin receptor α-subunit can be quantified by measuring the signal originating from this label.

[0030]

Any method may be used to bind the antibodies to the solid phase. For example, antibodies can be physically adsorbed to hydrophobic materials such as polystyrene. Alternatively, antibodies can be chemically bound to a variety of materials having functional groups on their surfaces. Furthermore, antibodies labeled with a binding ligand thereof can be bound to a solid phase by trapping the ligand with its binding partner. Combination of a binding ligand and its binding partner includes the avidin-biotin combination or such. The solid phase and antibodies can be conjugated at the same time when reacting the second antibody, or after this reaction.

Similarly, labeling of the second antibody does not have to be direct. More specifically, indirect labeling using binding reactions such as antibodies against antibodies, or avidin to biotin, is also possible.

[0031]

The concentration of insulin receptor α-subunit in a sample is determined based on the signal intensities measured for standard samples whose insulin receptor α-subunit concentrations are known.

[0032]

The immobilized antibodies and the second antibodies used for the heterogeneous immunoassays mentioned above may be any, as long as they recognize an insulin receptor α-subunit, or fragments comprising the antigen-binding site. Therefore, monoclonal antibodies, polyclonal, or a mixture or combination of both may be used. When both antibodies are monoclonal antibodies, combining monoclonal antibodies recognizing different epitopes is preferred.

[0033]

Since the antigen to be measured is sandwiched by antibodies, such heterogenous immunoassays are called sandwich methods. As sandwich methods excel in measurement sensitivity and reproducibility, they are one of the preferred principles of measurement in the present invention.

[0034]

The principles of competitive inhibition reactions can be applied to the heterogeneous immunoassays. More specifically, these are immunoassays based on the phenomenon that the insulin receptor α-subunit in a sample competitively inhibits the binding between an antibody and the insulin receptor α-subunit of a known concentration. The insulin receptor α-subunit concentration in the sample can be determined by labeling the insulin receptor α-subunit of known concentration and measuring the amount of the insulin receptor α-subunit that reacted (or did not react) with the antibody.

[0035]

A competitive reaction system is established by simultaneously reacting, with antibodies, antigens of a known concentration and antigens in a sample. Furthermore, analyses by an inhibitory reaction system are possible when antibodies are reacted with antigens of a known concentration after reacting with antigens in the sample. In both types of reaction systems, reaction systems that are superior in operability can be constructed by preparing either the antibodies or the antigens of known concentration (used as reagent components) as labeled components, and the other as the immobilized reagents.

[0036]

The labeling components used in such heterogeneous immunoassays include radioisotopes, fluorescent substances, light-emitting substances, substances having enzymatic activity, macroscopically observable substances, and magnetically observable substances. Specific examples of these labeling substances are shown below.

Substances having enzymatic activity:

peroxidase,
alkaline phosphatase,
urease, catalase,
glucose oxidase,
lactate dehydrogenase,
amylase, etc.

Fluorescent substances:

fluorescein isothiocyanate,
tetramethylrhodamine isothiocyanate,
substituted rhodamine isothiocyanate,
dichlorotriazine isothiocyanate, etc.

Radioisotopes:

tritium,
^{125}I,
^{181}I, etc.

Of these, enzymes or such non-radioactive labels are among advantageous labels in terms of safety, operability, sensitivity, and such. Enzymatic labels can be linked to antibodies or to an insulin receptor α-subunit by known methods such as the periodate method, or maleimide method.

[0037]

As the solid phase, beads, inner walls of a container, fine particles, porous carriers, magnetic particles, or such are used. Solids that have been fabricated using materials such as polystyrene, polycarbonate, polyvinyltoluene, polypropylene, polyethylene, polyvinyl chloride, nylon, polymethacrylate, latex, gelatin, agarose, glass, metal, ceramic, or such can be used for these solid phases. Solid materials to whose surface functional groups for chemically binding antibodies and such have been introduced are known. Known linking methods including chemical bonding such as poly-L-lysine or glutaraldehyde treatment, or physical adsorption can be applied for solid phases and antibodies (or antigens).

[0038]

Although the steps of separating the solid phase from the liquid phase as well as washing steps are required in all heterogeneous immunoassays exemplified herein, these steps

can easily be performed using immunochromatography, which is a variation of the sandwich method.

More specifically, antibodies to be immobilized are fixed onto porous carriers capable of transporting a sample solution by capillary action. Then a mixture of a sample comprising the insulin receptor α-subunit and labeled antibodies is migrated through this by capillary action. During this migration, the insulin receptor α-subunit reacts with the labeled antibodies, and when it comes into contact with the immobilized antibodies, is trapped at that position. Labeled antibodies that have not reacted with the insulin receptor α-subunit pass through without being trapped on the immobilized antibodies.

[0039]

As a result, presence of the insulin receptor α-subunit can be detected using as an indicator the signals of the labeled antibodies remaining at the position of the immobilized antibodies. If the labeled antibodies are preloaded on the upstream in the porous carrier, all reactions will be initiated and completed by simply dropping the sample solution, resulting in the construction of an extremely convenient reaction system. In immunochromatography, labeled components that can be distinguished macroscopically, such as colored particles, can be combined to construct an analytical device that does not even need a special reader.

[0040]

Next, homogeneous immunoassays will be described. In contrast to the heterogeneous immunoassay method that requires separation of the reaction solution as described above, the insulin receptor α-subunit can also be measured by homogeneous analysis methods. Using the homogeneous analysis method, antigen-antibody reaction products can be detected without separating them from the reaction solution.

[0041]

A representative homogeneous analysis method is an immunoprecipitation reaction in which antigenic substances are quantitatively analyzed by examining precipitates produced following the antigen-antibody reaction. Polyclonal antibodies are generally used for the immunoprecipitation reaction. When applying monoclonal antibodies, multiple types of monoclonal antibodies that bind to different epitopes of the insulin receptor α-subunit are preferably used. The products of precipitation reaction associated with the immunological reaction can be macroscopically observed, or they can be converted to numerical data by optical measurements.

[0042]

In contrast to these immunoassay methods utilizing the formation of immunological complexes in liquid phase, methods that perform the reaction in gels are also known. Such examples include Ouchterlony test, SRID method, and immunoelectrophoresis. In these

methods of analysis based on reactions in gels, clear precipitation lines can be observed by using multiple types of monoclonal antibodies.

[0043]

Immunological particle agglutination reaction, which uses as an indicator the agglutination of antibody-sensitized fine particles by antigens, is a common homogeneous analysis method. As in the aforementioned immunoprecipitation reaction, polyclonal antibodies or a combination of multiple types of monoclonal antibodies can be used in this method. Fine particles can be sensitized with antibodies through sensitization with a mixture of antibodies, or they can be prepared by mixing particles that have been separately sensitized with each type of antibody. Fine particles obtained in this manner gives matrix-like reaction products upon contact with the insulin receptor α-subunit. The reaction products can be detected as particle aggregates. Particle aggregation may be macroscopically observed, or may be converted into numerical data through optical measurements.

[0044]

Immunoassay methods based on energy transfer and enzyme channeling are known as examples of homogeneous immunoassays. In methods utilizing energy transfer, different optical labels that have donor/acceptor relationships are linked to each of a plurality of antibodies recognizing adjacent epitopes on an antigen. When immunological reactions take place, the donor and acceptor come closer to each other and energy transfer occurs, resulting in signal extinction or signals such as changes in fluorescence wavelength. On the other hand, enzyme channeling utilizes a combination of enzymes, which are related in that the reaction product of one enzyme is the substrate of the other, as a label for a plurality of antibodies bound to adjacent epitopes. When the enzymes come close to each other due to immunological reactions, the enzyme reactions are enhanced; therefore their binding can be detected as a change in enzyme reaction rates.

[0045]

The present invention also provides reagents that comprise antibodies recognizing the insulin receptor α-subunit and that are used for measuring free insulin receptor α-subunit in blood. The presence of free insulin receptor α-subunits in blood is a novel finding made by the present inventors. Therefore, the usefulness of antibodies recognizing the insulin receptor α-subunit as reagents for measuring the free insulin receptor α-subunit in the blood is also a novel discovery. Depending on the assay format, antibodies that constitute the measuring reagent of the present invention can be labeled or bound to a solid phase.

The above-exemplified labeled antibodies (or antigens) and immobilized antibodies (or antigens) that are necessary for the each immunoassay method can be prepared as kits by combining them with an insulin receptor α-subunit standard with a predetermined concentration ,

a buffer for dilution or washing, and such.

[0046]

Blood samples are used for the present invention's methods for measuring the insulin receptor α-subunit. Blood samples include whole blood and serum or plasma separated from whole blood. Whole blood can also be used as an analysis sample after disrupting blood cell components. Furthermore, blood samples can be diluted as necessary.

The presence of free-form insulin receptor α-subunits in blood is a novel finding made by the present inventors. The insulin receptor α-subunits in the blood are also found in healthy individuals. However, in experiments using mice, administration of the insulin receptor α-subunit into blood caused hyperglycemia and increase in the amount of insulin secretion. Therefore, insulin receptor α-subunits in blood are important as a risk factor for diabetes (type 2 diabetes mellitus), or as an exacerbation factor of type 2 diabetes mellitus. In addition, the information obtained by measuring the free insulin receptor α-subunit in blood in living bodies would be useful for evaluating the risk of diabetes in subjects.

[0047]

The present inventors showed that a free form of the insulin receptor α-subunit exists in blood. To measure the free form of the insulin receptor α-subunit by an immunoassay, antibodies recognizing the subunit are necessary. Additionally needed are antigens that are usable as a standard sample and have an antibody reactivity that is similar to that of free insulin receptor α-subunit in the living body. The present invention provides polynucleotides useful for producing insulin receptor α-subunits, which can be used as immunogens for obtaining such antibodies, or as standard samples.

[0048]

More specifically, the present invention relates to a polynucleotide described in any one of the following (a) to (b), and polypeptides encoded by the polynucleotides:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1;

(b) a polynucleotide encoding the amino acid sequence of SEQ ID NO: 2;

(c) a polynucleotide that hybridizes with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 under stringent conditions and encodes a secretory polypeptide that is immunologically equivalent to the insulin receptor α-subunit;

(d) a polynucleotide that has 90% or more homology to the nucleotide sequence of SEQ ID NO: 1 and encodes a secretory polypeptide that is immunologically equivalent to the insulin receptor α-subunit.

[0049]

The polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 described in (a) encodes amino acid sequences constituting each of the regions described below in the human

insulin receptor gene. The position of each of these regions in SEQ ID NO: 2 are shown within parenthesis. Furthermore, the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 1 is shown by an underline in Fig. 2.

signal peptide (-27 to -1);

α-subunit (1 to 735); and

a part of the β-subunit (736 to 926)

Those skilled in the art can synthesize such polynucleotides based on the nucleotide sequence shown in SEQ ID NO: 1. Alternatively necessary nucleotide sequences can be obtained from the cDNA of known insulin receptors. For example, in the Examples, a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 was obtained by digesting the human insulin receptor gene using restriction enzyme *Ssp*I.

[0050]

The present inventors discovered that when an amino acid sequence in which part of the β-subunit is added to the human insulin receptor α-subunit is expressed, the expression product is efficiently secreted to the outside of the cell. In general, receptor molecules expressed on the cell membrane is difficult to purify because removal of cell membrane components becomes an obstacle. In other cases, the removal of cell membrane components disables the maintenance of the receptor molecule structure. Therefore, expressing the receptor molecule as a secretory protein is a useful production technique.

[0051]

An objective of the present invention is to produce polypeptides useful as standard samples of the free form of the insulin receptor α-subunit or as immunogens. Recombinant proteins secreted outside the cell can be regarded as free molecules having the same existence form as in living bodies. That is, compared to cell membrane receptor molecules extracted from tissues, the polypeptides of this invention that are expressed as secretory proteins are preferred as standard samples or immunogens.

[0052]

The polynucleotide of the present invention includes the aforementioned polynucleotide of (c) or (d). In the present invention, immunological equivalence can be determined based on reactivity with antibodies. More specifically, when the reactivity of antibodies against the free insulin receptor α-subunit in the blood is absorbed by a certain protein, this protein can be said to be immunologically equivalent to the free insulin receptor α-subunit in blood. Furthermore, when a certain protein is expressed in suitable host cells, and if that protein is secreted into the culture supernatant of the cells, that protein can be proved to be a secretory protein. Typically, when the insulin receptor gene is expressed in transformants, the receptor molecule is localized on the cell membrane due to the function of the transmembrane domain of the β-subunit, and

detecting the α-subunit in the culture supernatant is difficult.

[0053]

There are no particular limitations on the number of amino acids that are mutated in the protein that is immunologically equivalent to the insulin receptor α-subunit, as long as the immunological equivalence is maintained. Regarding the insulin receptor α-subunit, the number of amino acids to be mutated is usually 100 amino acids or less, preferably 50 amino acids or less, more preferably 30 amino acids or less, and even more preferably 10 amino acids or less. Furthermore, there are no limitations on the site of mutation as long as the immunological equivalence is maintained.

[0054]

Amino acid mutations may be artificial or naturally-occurring mutations. When substituting amino acids, conservative substitution may be utilized. Generally, to maintain protein function, the amino acid used for the substitution preferably has characteristics similar to those of the amino acid before substitution. This type of amino acid residue substitution is called conservative substitution.

For example, Ala, Val, Leu, Ile, Pro, Met, Phe, and Trp are all categorized as non-polar amino acids, and have characteristics similar to each other. Examples of uncharged amino acids include Gly, Ser, Thr, Cys, Tyr, Asn, and Gln. Examples of acidic amino acids include Asp and Glu. Furthermore, basic amino acids include Lys, Arg, and His. Amino acids that constitute each of these groups respectively have characteristics similar to each other. Therefore, the substitution with another amino acid within the same group is likely to maintain the function of the protein.

Such proteins can be obtained by introducing mutations to the nucleotide sequence of SEQ ID NO: 1. Techniques for introducing mutations into a gene comprising a known nucleotide sequence are well known. Alternatively, a protein comprising a desired amino acid sequence can be prepared by chemical synthesis.

[0055]

As another method for isolating the aforementioned immunologically equivalent protein, hybridization screening can be utilized. For example, those skilled in the art can easily use a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof to isolate DNAs highly homologous thereto. Subsequent selection of DNAs that encode proteins immunologically equivalent to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2 from thus-isolated DNAs can also be readily carried out by those skilled in the art.

As described, polypeptides that are encoded by DNAs hybridizing with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 and are immunologically equivalent to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2 are also included in the

polypeptides of the present invention. Those skilled in the art can appropriately select hybridization stringencies for isolating DNAs encoding the immunologically equivalent polypeptides.

[0056]

Specific hybridization conditions include, for example, conditions of 5x SSC, in the absence of formamide, at 25°C. Hybridization is preferably performed under conditions of 6x SSC, 40% formamide, at 25°C; and more preferably under conditions of 5x SSC, 50% formamide, at 40°C. Washing after hybridization is performed for example under conditions of 2x SSC, at 37°C, preferably 1x SSC, at 55°C, and more preferably 1x SSC, at 60°C.

[0057]

Instead of hybridization screening, the PCR method using oligonucleotides complementary to a portion of the nucleotide sequence of SEQ ID NO: 1 as primers can be applied to isolate the polynucleotide of this invention.

[0058]

Polynucleotides that can be isolated by hybridization screening or PCR described above, and encode a polypeptide that is immunologically equivalent to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2, are usually highly homologous to the nucleotide sequence of SEQ ID NO: 1. In the present invention, highly homologous means having sequence identity of at least 20% or more, preferably 30% or more, more preferably 40% or more, even more preferably 60% or more, and yet even more preferably 80% or more, throughout the entire polynucleotide and not just a portion thereof. Algorithms for determining nucleotide sequence homology are known (Takashi Miyata *et al.* "Computer-Assisted Genetic Homology Analysis" (Genetic Research Methods I, Tokyo Kagaku-Dojin)).

[0059]

The polynucleotides of the present invention can be used, for example, for producing recombinant proteins. *E. coli*, yeast, insect cells, animal cells, and such can be used as hosts for producing the polypeptides of the present invention as recombinant proteins. Suitable vectors are respectively determined according to the host cells. For example, the following expression vectors can be used for each type of host cell.

E. coli: pGEX5X-3 (Pharmacia), and such
yeast: pYES2 (Invitrogen), and such
insect cells: pVL1392 (Invitrogen), and such
animal cells: pRc/CMV2 (Invitrogen), and such

Methods for introducing these vectors into hosts known by those skilled in the art include biological methods, physical methods, and chemical methods. Biological methods include methods that use virus vectors. Physical methods include electroporation, GENEGUN

method, or microinjection. Chemical methods include lipofection, calcium phosphate method, and DEAE-Dextran method.

Recombinant proteins produced in hosts can be purified by a discretionary method. Specifically, methods that utilize ion exchange column, affinity column, and such are generally used. The method for expressing recombinant proteins as fusion proteins with GST or 6x His to facilitate detection and purification are also known.

[0060]

The polynucleotides of (a) to (d) described above can all be used to express the insulin receptor α-subunit (or an immunologically equivalent polypeptide) as a secretory protein. The extracellularly secreted insulin receptor α-subunit can be easily collected from the culture supernatant. Alternatively, the culture supernatant can be directly used as a standard sample or an immunogen.

[0061]

For example, standard samples for measuring free insulin receptor α-subunits in blood can be produced as follows. First, the culture supernatant of the aforementioned transformant is collected. The collected culture supernatant, is used as it is, or after purifying the desired expression product, for assaying the expression product amount. When the expression product is purified as a pure protein, the amount of the expression product is determined by measuring the concentration of that protein. Alternatively, if there are any contaminants as in a culture supernatant, the amount of the desired expression product can be determined by isolating the product by various chromatographic techniques or electrophoresis. Once the amount of the expression product is determined, the supernatant or the purified protein can be utilized as a standard sample.

[0062]

The amino acid sequence of SEQ ID NO: 2 is an amino acid sequence derived from a human insulin receptor. When a polypeptide comprising an amino acid sequence different from this amino acid sequence is used as an expression product, the differences in molecular mass can be corrected based on differences in the amino acid sequences of the two.

[0063]

In the present invention, a "standard sample of a certain protein" refers to a sample in which the amount of this protein has been determined in advance. Standard samples can be serially diluted as necessary. Signals are measured for these serially diluted samples by the aforementioned immunoassays. The relationship between the concentrations of the protein in the standard samples and the measured signals can be expressed as a standard curve (or a calibration curve). Based on the standard curve made in this manner, the concentration of the substance to be measured in the sample can be determined from signals obtained from actual test

samples. Alternatively, by formulating a regression equation based on the measurement results of the serial dilutions, and by assigning the values measured from the test samples, the concentration of the substance to be measured in the sample can be determined.

Hereinafter, the present invention will be described more specifically with reference to Examples.

[0064]

[Examples]

1. Construction of human insulin receptor α-subunit cDNA

cDNA for causing secretion of insulin receptor α-subunit into the culture supernatant from CHO cells were constructed. The structures of the cDNAs constructed in the present example are shown in Fig. 1. Each of the cDNAs was obtained by using pcDL1-HIR717 (Ebina *et al.* Cell. 40, 747-758, 1985) comprising the full-length cDNA (NM_000208) of the human insulin receptor and digesting it at different positions. Each of the cDNAs has the following structures, respectively.

[0065]

CHO-HIR: insulin receptor α-subunit + β-subunit

CHO-HIR(α): insulin receptor α-subunit alone

CHO-HIR(PstI): α-subunit and amino acids 1 to 150 in the N terminal side of the β-subunit

CHO-HIR(SspI): α-subunit and amino acids 1 to 191 in the N terminal side of the β-subunit

The β-subunit transmembrane domain is in positions 195 to 217 from the N-terminal side. This means that CHO-HIR(PstI) and CHO-HIR(SspI) both include a portion of the β-subunit, but lack the transmembrane domain. In Fig. 2, the boxed part within the β-subunit amino acid sequence (the C-terminal side part in lower-case letters) corresponds to the transmembrane region.

Each of the cDNAs was inserted into the animal cell expression vector pCXN2 to obtain expression plasmids of the hIR and its variants. Each restriction enzyme was purchased from Takara (Otsu, Japan) and New England BioLabs (Beverly, MA).

[0066]

2. Culturing and gene transfer

Chinese hamster ovary (CHO) cells were cultured in a 5% CO_2 incubator using F-12 Nutrient Mixture (Ham's F-12, Invitrogen, Carlsbad, CA) media. 10 μg of each expression plasmid obtained in 1 was linearized using the restriction enzyme *Sca*I, and this was introduced into CHO cells by electroporation with 0.5 μg of blasticidin (pSV2-bsr, Funakoshi, Tokyo, Japan). 24 hours after transfection, the medium was changed to 10 μg/mL blasticidin-resistant F-12 medium, and two weeks later, the remaining colonies were isolated. Expression of hIR was confirmed by polyacrylamide electrophoresis and Western blotting using anti-IRa

antibodies.

[0067]

3. Purification of the α-subunit

Clones showing high expression were amplified in F-12 medium containing 10% FCS, and cultured in 150-mm cell culture dishes (Corning). After culturing to 100% confluency, the cells were washed three times with HEPES buffer [20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 1 mM $CaCl_2$] and the medium was exchanged with 20 mL of serum-free medium (CHO-S-SFM II DPM, Invitrogen) per dish.

[0068]

After culturing for four days, the medium was collected, cell components were removed by centrifugation (1,300 x g, 10 minutes, 4°C), and then the supernatant was collected. 6 μL (50% suspension) of wheat germ lectin (WGA) agarose (Amersham) was added per 1 mL of the supernatant, and this was rotated at 4°C for two hours for adsorption. The agarose was washed five times. The first and fifth washes were carried out using HEPES buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100]. The salt concentration of the washing solution for the second to fourth washes was set to 500 mM. The adsorbed protein was eluted using HEPES buffer containing 0.2 M *N*-acetylglucosamine. The eluted sample was added to anti-insulin receptor antibody (α-subunit antibody, Immunotech 0365) column (bed volume; 0.5 mL) and allowed to adsorb while rotating at 4°C for one hour. The column was washed three times with ten times the bed volume of HEPES buffer, and in this case as well, the salt concentration for the second wash was increased to 500 mM. Using sodium borate buffer containing 1.5 M $MgCl_2$, 200-μL fractions were collected.

[0069]

The degree of purification of the insulin receptor α-subunit in each fraction was evaluated as follows. First, 20 μL of each fraction was withdrawn and subjected to 7.5% SDS-PAGE and silver staining. A photograph of the stained gel is shown in Fig. 3. A protein having a molecular mass corresponding to the insulin receptor α-subunit was confirmed to be isolated as a nearly pure protein (the band indicated as IRα). The stained gel was scanned with a transmission scanner and evaluated using NIH image software. The protein concentration was determined by the Bradford method using Protein Assay Dye Reagent (BioRad, Hercules, CA) with BSA as the standard.

[0070]

4. Production of anti-human insulin receptor α-subunit antibodies

Using the purified human insulin receptor α-subunit, rabbits (Japanese white rabbits, female, 3.5 kg) were immunized subcutaneously (at approximately ten sites, once a week). After five immunizations, a small amount of blood was collected from the ear veins, and the

serum was separated to check the antibody titer by ELISA.

[0071]

First, human insulin receptor α-subunit was dissolved in 1/100 M physiological phosphate-buffered saline (PBS) to prepare a 0.1 mg/mL solution, and 100 μL aliquots of this solution was added to Nunc 96-well microplates "Maxisorp". After letting this stand at room temperature (20 to 25°C) for three hours, the solution inside the wells were removed by suction, and 30 μL of PBS containing 5% bovine serum albumin was added. This was left undisturbed for approximately 18 hours at 4°C, and the unreacted parts in the cups were blocked. After removing the blocking solution, this plate was washed three times with 300 μL of PBS and used as the plates for ELISA.

[0072]

Antiserum diluted with PBS was further diluted to produce a series of dilutions. 100 μL of the diluted serum was added to each well of the ELISA plate. After leaving this undisturbed at room temperature (20 to 25°C) for one hour, the reaction solution was removed, and then the plate was washed four times with 30 μL of PBS. Next, 100 μL of the diluted peroxidase-labeled anti-rabbit IgG (Medical and Biological Laboratories Co., Ltd.) was added. After letting this react while standing at room temperature (20 to 25°C) for one hour, washing was done four times with 30 μL of PBS.

[0073]

100 μL of a solution of 3,3',5,5'-tetramethylbenzidine with hydrogen peroxide was added to the washed wells as the chromogenic substrate. After letting this react for a certain length of time, 1 N sulfuric acid was added to stop the reaction, and the absorbance at wavelength of 450 nm was measured. The results of the measurements are shown in the graph of Fig. 4. As is clear from this result, the obtained antiserum exhibited sufficient antibody titer. From rabbits that showed sufficient antibody titer in this manner, 70 mL of blood was collected from the ear veins and approximately 30 mL of antiserum was obtained. Furthermore, from the polyclonal solution obtained in this manner, IgG fraction was purified using a DEAE cellulose column.

[0074]

5. Preparation of labeled antibodies

Anti-rabbit IgG monoclonal antibodies (2B9 5 μg/mL) dissolved in 0.1 M carbonate buffer (pH 8.5) and NHS-LC-BIOTIN (PIERCE, 25 μg/mL) were mixed and stirred at room temperature for four hours using a stirrer. IgG and NHS-LC-BIOTIN were mixed at a mole ratio of 1:60. After stirring, this solution was dialyzed against physiological phosphate buffered saline (PBS) to obtain biotin-labeled antibodies.

[0075]

6. Construction of an ELISA system for the human insulin receptor α-subunit

Anti-human insulin receptor α-subunit antibodies were diluted and prepared to a concentration of 40 g/mL to 80 g/mL with 0.1 M carbonate buffer (pH 9.6). 100-μL aliquot of the diluted antibody solution was placed into each well of a 96-well microplate (NUNC, Immuno Break Apart Modules Maxisorp #473768). The microplate was left to stand overnight in a humid container at 2 to 8°C to bind the antibodies.

[0076]

After incubation, the antibody solution was discarded, and the plate was washed twice with PBS. Excess moisture was removed, and blocking solution (PBS containing 1% BSA and 0.1% NaN_3) was added in 200-μL aliquots per well. Blocking was carried out by placing the microplate in a humid container overnight at 2 to 8°C. After blocking, the blocking solution was discarded and excess moisture was removed. Furthermore, the plates were air-dried, and were stored until use in an aluminum-coated bag with a drying agent.

[0077]

6. Evaluation of the ELISA system

The purified human insulin receptor α-subunit was diluted with a sample dilution buffer (20 mM Tris-HCl, 150 mM NaCl, 1% BSA, 10% normal mouse serum, 25 mg/mL MAK33, 0.1% NaN_3, 1% bovine γ-globulin, 0.056% Tween20, pH 7.5) and used as standards. Samples were two-fold diluted with the sample dilution solution and placed into each well of the antibody-sensitized microplate in 100-μL aliquots, and this was allowed to react for three hours at room temperature to form antibody-antigen complexes.

[0078]

After the reaction, the wells were washed five times with a washing buffer (PBS + 0.05% Tween20). Excess moisture was removed, then 100-μL aliquots of the biotin-labeled antibody diluted with the sample dilution buffer was added to each well, and this was allowed to react at room temperature for three hours. After the reaction, the wells were washed five times using the same washing buffer. After removing excess moisture, avidin-labeled peroxidase diluted with avidin HRP dilution buffer (20 mM Tris-HCl, 150 mM NaCl, 1% BSA, 0.15% Proclin, pH 7.5) was added to each well in 100-μL aliquots, and this was allowed to react at room temperature for three hours. These reactions lead to the formation of an antibody/antigen/biotinylated antibody/avidin-labeled peroxidase complex.

[0079]

The reacted wells were washed five times with the washing buffer. After removing excess moisture, 100 μL of TMB chromogenic substrate (MOSS, TMBH-100) was added per well. After letting this react at room temperature for approximately 20 minutes for color development, 100 μL of 1 N sulfuric acid was added per well to stop the color development.

After this, absorbance was measured at wavelength of 450 nm. The concentration of insulin receptor α-subunit in the samples was read from the calibration curve prepared from the absorbance of the standards. The ELISA system constructed as described above was confirmed to be able to measure the insulin receptor α-subunit.

[0080]

7. Effects of administering the insulin receptor α-subunit

Eight to ten-week-old male mice were fasted for 16 hours and anesthetized using pentobarbital. Next, 100 ng of the purified insulin receptor α-subunit was administered from the cervical vein. The insulin receptor α-subunit was administered in a form dissolved in 50 μL of physiological saline containing 0.1% BSA. Blood was withdrawn from insulin receptor α-subunit-administered mice at various time points through the tail vein, and the blood sugar level was measured. As a control, 50 μL of physiological saline containing 0.1% BSA alone was administered to mice in the same manner.

[0081]

The results are shown in Fig. 5. The blood sugar level in the control mice gradually decreased due to continued fasting. On the other hand, in mice that received the insulin receptor α-subunit administration into the blood, over-time increase in the blood glucose level was observed. This may have occurred as a result of the binding of the administered insulin receptor α-subunit with insulin, which inhibited insulin action.

[0082]

8. Effects of insulin receptor α-subunit administration (glucose loading)

Ten minutes after administering the insulin receptor α-subunit under the same conditions as in 7, glucose (2 g/kg body weight) was administered intraperitoneally. After glucose administration, blood was collected from the tail vein at different time intervals, and the blood sugar levels were measured. As a control, the same amount of glucose was administered to mice that had been given only physiological saline containing 0.1% BSA (50 μL) by the same method.

[0083]

The results are shown in Fig. 6. Abnormal glucose tolerance was confirmed in glucose tolerance tests upon administration of insulin receptor α-subunit. These two results indicate that when the insulin receptor α-subunit exists in blood, it is very likely to act as an exacerbation factor for diabetes through its binding to insulin to inhibit insulin action and increase blood glucose level.

[0084]

[Effects of the Invention]

The present invention provides methods for measuring free insulin receptor α-subunit in

blood. Until now, the presence of free insulin receptor α-subunit in blood has not been confirmed. Furthermore, methods for measuring it have not been established. The present inventors elucidated that the free insulin receptor α-subunit in blood obstructs insulin action. Therefore, measuring the free insulin receptor α-subunit in blood is useful for evaluating the risk for diabetes.

The present invention also provides free-form insulin receptor α-subunits, which are useful for measuring free insulin receptor α-subunit, and methods for producing them. The free-form α-subunits obtainable by the present invention can be utilized as standard samples and immunogens.

[0085]

[Sequence Listing]

SEQUENCE LISTING

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Toshiyuki, OBATA
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680 685 690
aag gag ctg gag gag tcc tcg ttt agg aag acg ttt gag gat tac ctg 2208
Lys Glu Leu Glu Glu Ser Ser Phe Arg Lys Thr Phe Glu Asp Tyr Leu
695 700 705
cac aac gtg gtt ttc gtc ccc aga aaa acc tct tca ggc act ggt gcc 2256
His Asn Val Val Phe Val Pro Arg Lys Thr Ser Ser Gly Thr Gly Ala
710 715 720 725
gag gac cct agg cca tct cgg aaa cgc agg tcc ctt ggc gat gtt ggg 2304
Glu Asp Pro Arg Pro Ser Arg Lys Arg Arg Ser Leu Gly Asp Val Gly
730 735 740

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            745                 750                 755
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Ser Thr Ser Val Pro Thr Ser Pro Glu Glu His Arg Pro Phe Glu Lys
        760                 765                 770
gtg gtg aac aag gag tcg ctg gtc atc tcc ggc ttg cga cac ttc acg     2448
Val Val Asn Lys Glu Ser Leu Val Ile Ser Gly Leu Arg His Phe Thr
    775                 780                 785
ggc tat cgc atc gag ctg cag gct tgc aac cag gac acc cct gag gaa     2496
Gly Tyr Arg Ile Glu Leu Gln Ala Cys Asn Gln Asp Thr Pro Glu Glu
790                 795                 800                 805
cgg tgc agt gtg gca gcc tac gtc agt gcg agg acc atg cct gaa gcc     2544
Arg Cys Ser Val Ala Ala Tyr Val Ser Ala Arg Thr Met Pro Glu Ala
                810                 815                 820
aag gct gat gac att gtt ggc cct gtg acg cat gaa atc ttt gag aac     2592
Lys Ala Asp Asp Ile Val Gly Pro Val Thr His Glu Ile Phe Glu Asn
            825                 830                 835
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Asn Val Val His Leu Met Trp Gln Glu Pro Lys Glu Pro Asn Gly Leu
        840                 845                 850
atc gtg ctg tat gaa gtg agt tat cgg cga tat ggt gat gag gag ctg     2688
Ile Val Leu Tyr Glu Val Ser Tyr Arg Arg Tyr Gly Asp Glu Glu Leu
    855                 860                 865
cat ctc tgc gtc tcc cgc aag cac ttc gct ctg gaa cgg ggc tgc agg     2736
His Leu Cys Val Ser Arg Lys His Phe Ala Leu Glu Arg Gly Cys Arg
870                 875                 880                 885
ctg cgt ggg ctg tca ccg ggg aac tac agc gtg cga atc cgg gcc acc     2784
Leu Arg Gly Leu Ser Pro Gly Asn Tyr Ser Val Arg Ile Arg Ala Thr
                890                 895                 900
tcc ctt gcg ggc aac ggc tct tgg acg gaa ccc acc tat ttc tac gtg     2832
Ser Leu Ala Gly Asn Gly Ser Trp Thr Glu Pro Thr Tyr Phe Tyr Val
            905                 910                 915
aca gac tat tta gac gtc ccg tca aat                                2859
Thr Asp Tyr Leu Asp Val Pro Ser Asn
        920                 925
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<400> 2

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Ala Val Ala Ala Leu Leu Leu Gly Ala Ala Gly His Leu Tyr Pro Gly
    -10                 -5                  -1  1               5
Glu Val Cys Pro Gly Met Asp Ile Arg Asn Asn Leu Thr Arg Leu His
                10                  15                  20
Glu Leu Glu Asn Cys Ser Val Ile Glu Gly His Leu Gln Ile Leu Leu
            25                  30                  35
Met Phe Lys Thr Arg Pro Glu Asp Phe Arg Asp Leu Ser Phe Pro Lys
        40                  45                  50
Leu Ile Met Ile Thr Asp Tyr Leu Leu Leu Phe Arg Val Tyr Gly Leu
    55                  60                  65
Glu Ser Leu Lys Asp Leu Phe Pro Asn Leu Thr Val Ile Arg Gly Ser
70                  75                  80                  85
Arg Leu Phe Phe Asn Tyr Ala Leu Val Ile Phe Glu Met Val His Leu
                90                  95                  100
Lys Glu Leu Gly Leu Tyr Asn Leu Met Asn Ile Thr Arg Gly Ser Val
            105                 110                 115
Arg Ile Glu Lys Asn Asn Glu Leu Cys Tyr Leu Ala Thr Ile Asp Trp
        120                 125                 130
Ser Arg Ile Leu Asp Ser Val Glu Asp Asn His Ile Val Leu Asn Lys
    135                 140                 145
Asp Asp Asn Glu Glu Cys Gly Asp Ile Cys Pro Gly Thr Ala Lys Gly
150                 155                 160                 165
Lys Thr Asn Cys Pro Ala Thr Val Ile Asn Gly Gln Phe Val Glu Arg
                170                 175                 180
Cys Trp Thr His Ser His Cys Gln Lys Val Cys Pro Thr Ile Cys Lys
            185                 190                 195
Ser His Gly Cys Thr Ala Glu Gly Leu Cys Cys His Ser Glu Cys Leu
        200                 205                 210
Gly Asn Cys Ser Gln Pro Asp Asp Pro Thr Lys Cys Val Ala Cys Arg
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215 220 225
Asn Phe Tyr Leu Asp Gly Arg Cys Val Glu Thr Cys Pro Pro Pro Tyr
230 235 240 245
Tyr His Phe Gln Asp Trp Arg Cys Val Asn Phe Ser Phe Cys Gln Asp
250 255 260
Leu His His Lys Cys Lys Asn Ser Arg Arg Gln Gly Cys His Gln Tyr
265 270 275
Val Ile His Asn Asn Lys Cys Ile Pro Glu Cys Pro Ser Gly Tyr Thr
280 285 290
Met Asn Ser Ser Asn Leu Leu Cys Thr Pro Cys Leu Gly Pro Cys Pro
295 300 305
Lys Val Cys His Leu Leu Glu Gly Glu Lys Thr Ile Asp Ser Val Thr
310 315 320 325
Ser Ala Gln Glu Leu Arg Gly Cys Thr Val Ile Asn Gly Ser Leu Ile
330 335 340
Ile Asn Ile Arg Gly Gly Asn Asn Leu Ala Ala Glu Leu Glu Ala Asn
345 350 355
Leu Gly Leu Ile Glu Glu Ile Ser Gly Tyr Leu Lys Ile Arg Arg Ser
360 365 370
Tyr Ala Leu Val Ser Leu Ser Phe Phe Arg Lys Leu Arg Leu Ile Arg
375 380 385
Gly Glu Thr Leu Glu Ile Gly Asn Tyr Ser Phe Tyr Ala Leu Asp Asn
390 395 400 405
Gln Asn Leu Arg Gln Leu Trp Asp Trp Ser Lys His Asn Leu Thr Thr
410 415 420
Thr Gln Gly Lys Leu Phe Phe His Tyr Asn Pro Lys Leu Cys Leu Ser
425 430 435
Glu Ile His Lys Met Glu Glu Val Ser Gly Thr Lys Gly Arg Gln Glu
440 445 450
Arg Asn Asp Ile Ala Leu Lys Thr Asn Gly Asp Lys Ala Ser Cys Glu
455 460 465
Asn Glu Leu Leu Lys Phe Ser Tyr Ile Arg Thr Ser Phe Asp Lys Ile
470 475 480 485
Leu Leu Arg Trp Glu Pro Tyr Trp Pro Pro Asp Phe Arg Asp Leu Leu
490 495 500
Gly Phe Met Leu Phe Tyr Lys Glu Ala Pro Tyr Gln Asn Val Thr Glu

505 510 515
Phe Asp Gly Gln Asp Ala Cys Gly Ser Asn Ser Trp Thr Val Val Asp
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Ile Asp Pro Pro Leu Arg Ser Asn Asp Pro Lys Ser Gln Asn His Pro
535 540 545
Gly Trp Leu Met Arg Gly Leu Lys Pro Trp Thr Gln Tyr Ala Ile Phe
550 555 560 565
Val Lys Thr Leu Val Thr Phe Ser Asp Glu Arg Arg Thr Tyr Gly Ala
570 575 580
Lys Ser Asp Ile Ile Tyr Val Gln Thr Asp Ala Thr Asn Pro Ser Val
585 590 595
Pro Leu Asp Pro Ile Ser Val Ser Asn Ser Ser Ser Gln Ile Ile Leu
600 605 610
Lys Trp Lys Pro Pro Ser Asp Pro Asn Gly Asn Ile Thr His Tyr Leu
615 620 625
Val Phe Trp Glu Arg Gln Ala Glu Asp Ser Glu Leu Phe Glu Leu Asp
630 635 640 645
Tyr Cys Leu Lys Gly Leu Lys Leu Pro Ser Arg Thr Trp Ser Pro Pro
650 655 660
Phe Glu Ser Glu Asp Ser Gln Lys His Asn Gln Ser Glu Tyr Glu Asp
665 670 675
Ser Ala Gly Glu Cys Cys Ser Cys Pro Lys Thr Asp Ser Gln Ile Leu
680 685 690
Lys Glu Leu Glu Glu Ser Ser Phe Arg Lys Thr Phe Glu Asp Tyr Leu
695 700 705
His Asn Val Val Phe Val Pro Arg Lys Thr Ser Ser Gly Thr Gly Ala
710 715 720 725
Glu Asp Pro Arg Pro Ser Arg Lys Arg Arg Ser Leu Gly Asp Val Gly
730 735 740
Asn Val Thr Val Ala Val Pro Thr Val Ala Ala Phe Pro Asn Thr Ser
745 750 755
Ser Thr Ser Val Pro Thr Ser Pro Glu Glu His Arg Pro Phe Glu Lys
760 765 770
Val Val Asn Lys Glu Ser Leu Val Ile Ser Gly Leu Arg His Phe Thr
775 780 785
Gly Tyr Arg Ile Glu Leu Gln Ala Cys Asn Gln Asp Thr Pro Glu Glu

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790                 795                 800                 805
Arg Cys Ser Val Ala Ala Tyr Val Ser Ala Arg Thr Met Pro Glu Ala
                810                 815                 820
Lys Ala Asp Asp Ile Val Gly Pro Val Thr His Glu Ile Phe Glu Asn
            825                 830                 835
Asn Val Val His Leu Met Trp Gln Glu Pro Lys Glu Pro Asn Gly Leu
        840                 845                 850
Ile Val Leu Tyr Glu Val Ser Tyr Arg Arg Tyr Gly Asp Glu Glu Leu
    855                 860                 865
His Leu Cys Val Ser Arg Lys His Phe Ala Leu Glu Arg Gly Cys Arg
870                 875                 880                 885
Leu Arg Gly Leu Ser Pro Gly Asn Tyr Ser Val Arg Ile Arg Ala Thr
                890                 895                 900
Ser Leu Ala Gly Asn Gly Ser Trp Thr Glu Pro Thr Tyr Phe Tyr Val
            905                 910                 915
Thr Asp Tyr Leu Asp Val Pro Ser Asn
        920                 925
```

[Brief Description of the Drawings]

[Fig. 1] Fig. 1 is a diagram showing the relationship between amino acid sequences encoded by the cDNAs used for expressing the insulin receptor α-subunit in CHO cells in the Examples, and the full-length amino acid sequence of the insulin receptor.

[Fig. 2] Fig. 2 shows the amino acid sequences of each subunit in the amino acid sequence of the insulin receptor precursor protein, and the relationship with the amino acid sequence of the recombinant expressed in the Examples. In the figure, capital letters indicate α-subunit, and the amino acid sequences shown in lower-case letters at the N-terminal and C-terminal side of the α-subunit are those of the signal peptide and β-subunit, respectively. In the amino acid sequence of the β-subunit, the boxed portion is the transmembrane (TM) region. The amino acid sequence encoded by the *Ssp*I-digested fragment shown in Fig. 1 is underlined.

[Fig. 3] Fig. 3 is a photograph showing the purification result of the insulin receptor α-subunit using an anti-insulin receptor α-subunit antibody column. The insulin receptor α-subunit adsorbed to the anti-insulin receptor antibody (α-subunit antibody, Immunotech 0365) column was eluted using sodium borate buffer containing 1.5 M $MgCl_2$, and the eluate was collected as 200-μL fractions. From each fraction, 20 μL was withdrawn and subjected to 7.5% SDS-PAGE and then silver stained.

[Fig .4] Fig. 4 shows the standard curve prepared in the Examples for the insulin

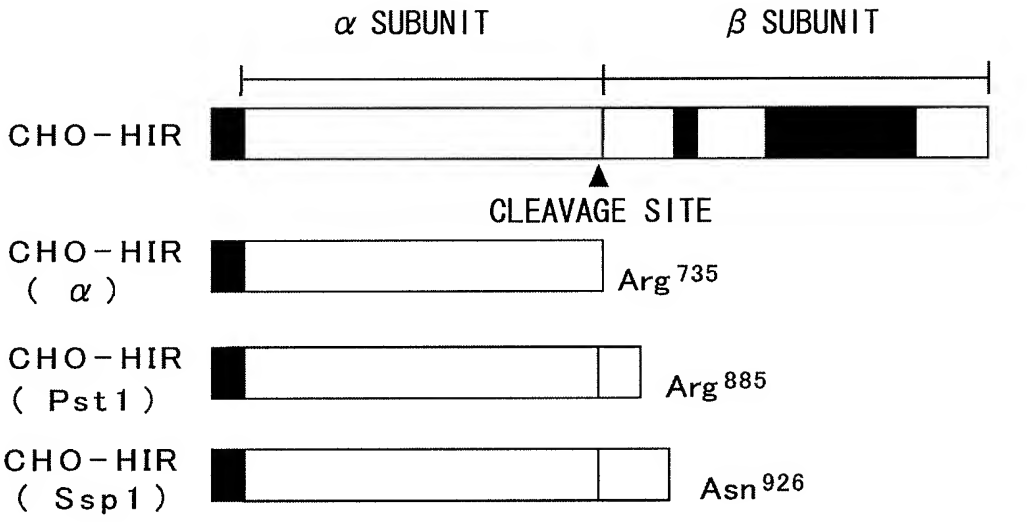
receptor α-subunit. In the figure, the vertical axis indicates the absorbance at 450 nm, and the horizontal axis indicates the concentration of the insulin receptor α-subunit (ng/mL) in the sample.

[Fig. 5] Fig. 5 shows the over-time changes in blood glucose level in mice to which the insulin receptor α-subunit has been administered. In the figure, the vertical axis indicates the blood glucose level (mg/mL), and the horizontal axis indicates the time elapsed (in minutes) where the time of insulin receptor α-subunit administration is set as -10.

[Fig. 6] Fig. 6 shows the over-time changes in blood glucose level in mice to which a glucose load has been given 10 minutes after administration of the insulin receptor α-subunit. In the figure, the vertical axis indicates the blood glucose level (mg/mL), and the horizontal axis indicates the time elapsed (in minutes) where the time of insulin receptor α-subunit administration is set as -10.

[Document Name] Drawings

[Fig. 1]



[Fig. 2]


Signal peptide
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-27 1
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VIFEMVHLKELGLYNLMNITRGSVRIEKNNELCYLATIDWSRILDSVEDNHIVLNKDDNE
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CHQYVIHNNKCIPECPSGYTMNSSNLLCTPCLGPCPKVCHLLEGEKTIDSVTSAQELRGC
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EIGNYSFYALDNQNLRQLWDWSKHNLTTTQGKLFFHYNPKLCLSEIHKMEEVSGTKGRQE
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NVTEFDGQDACGSNSWTVVDIDPPLRSNDPKSQNHPGWLMRGLKPWTQYAIFVKTLVTFS
DERRTYGAKSDIIYVQTDATNPSVPLDPISVSNSSSQIILKWKPPSDPNGNITHYLVFWE
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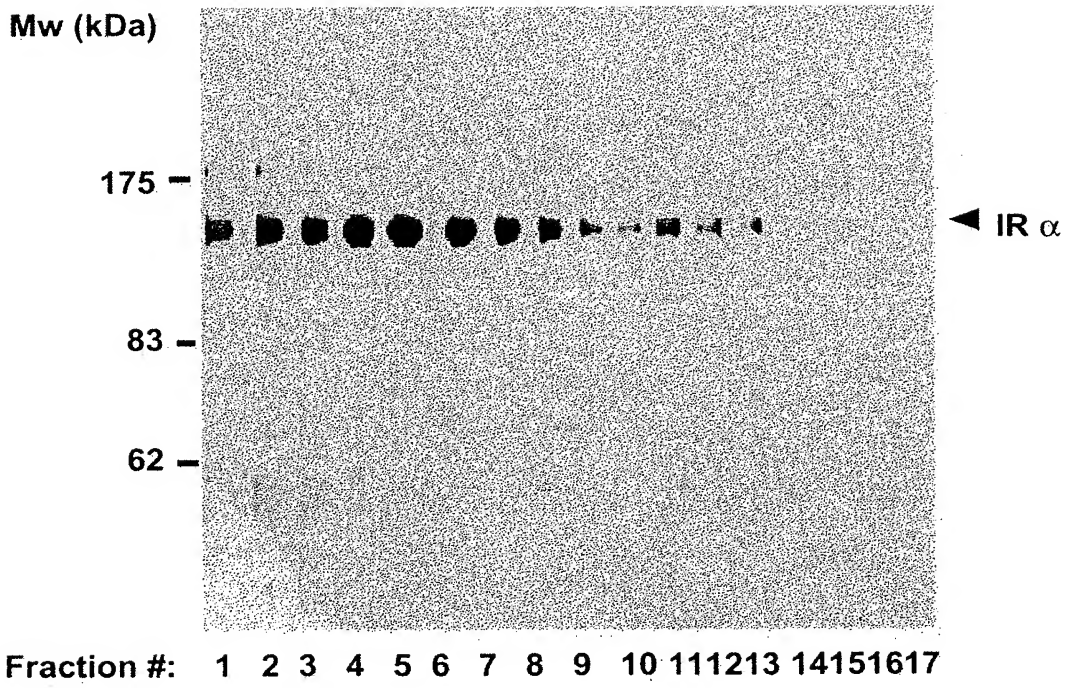
ps

[Fig. 3]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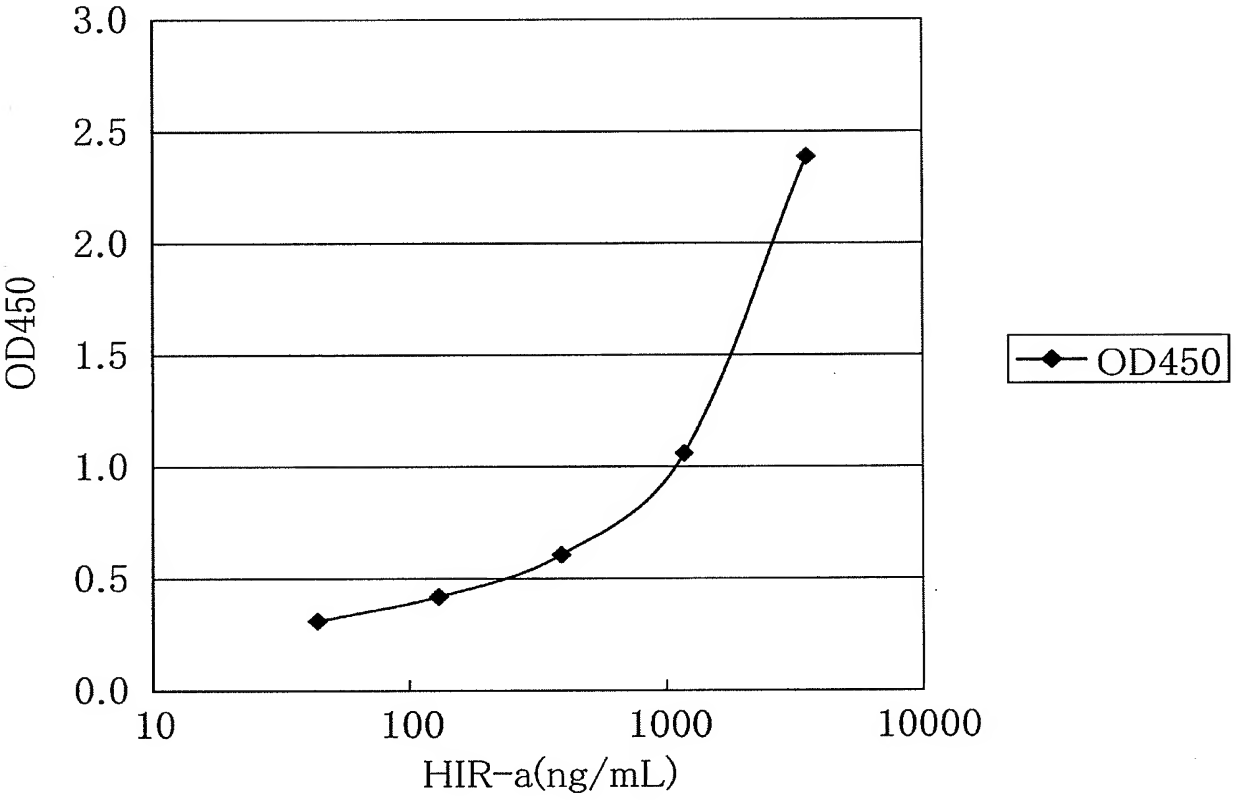
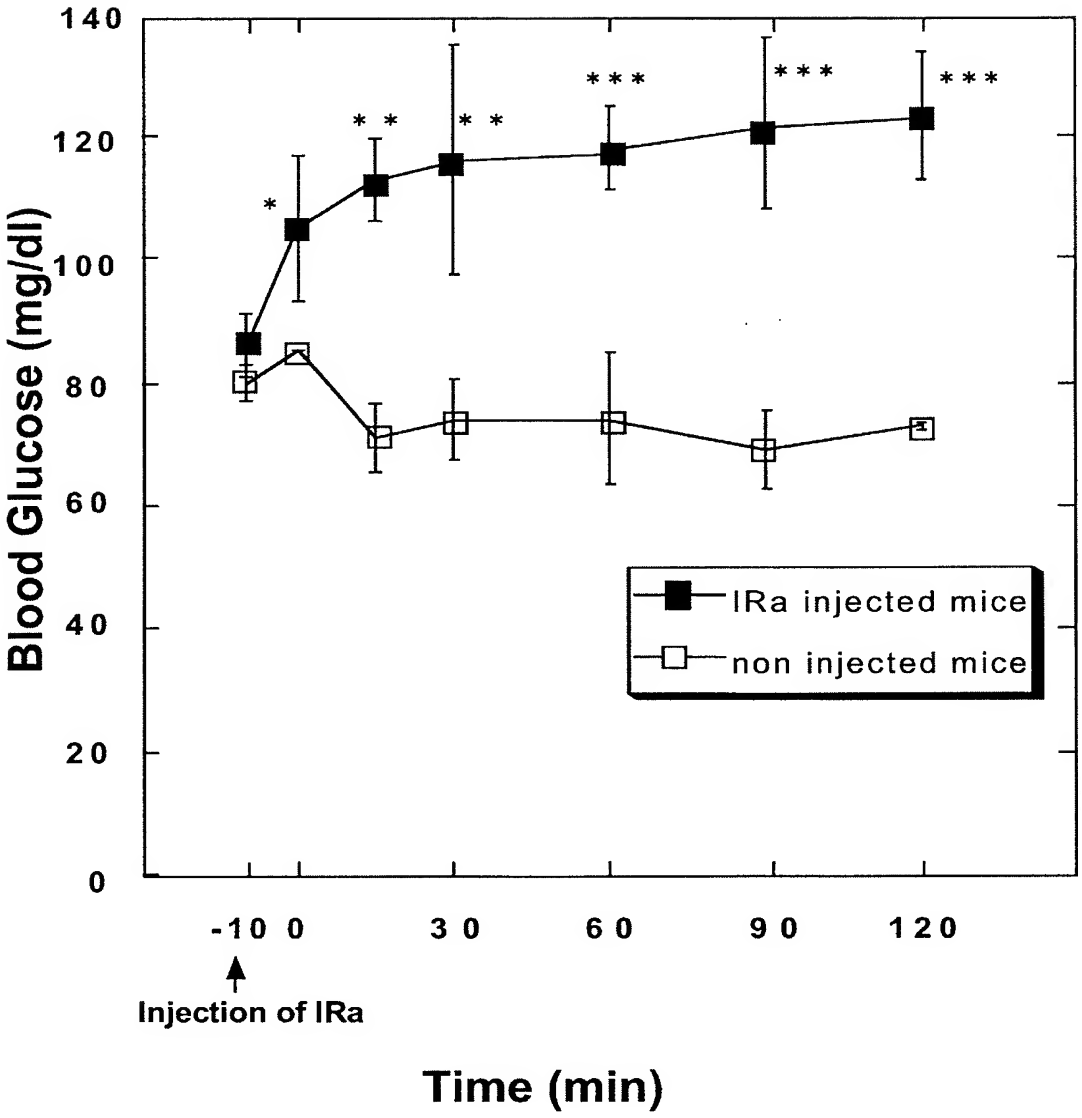
175
83
62
IR α
Fraction #: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

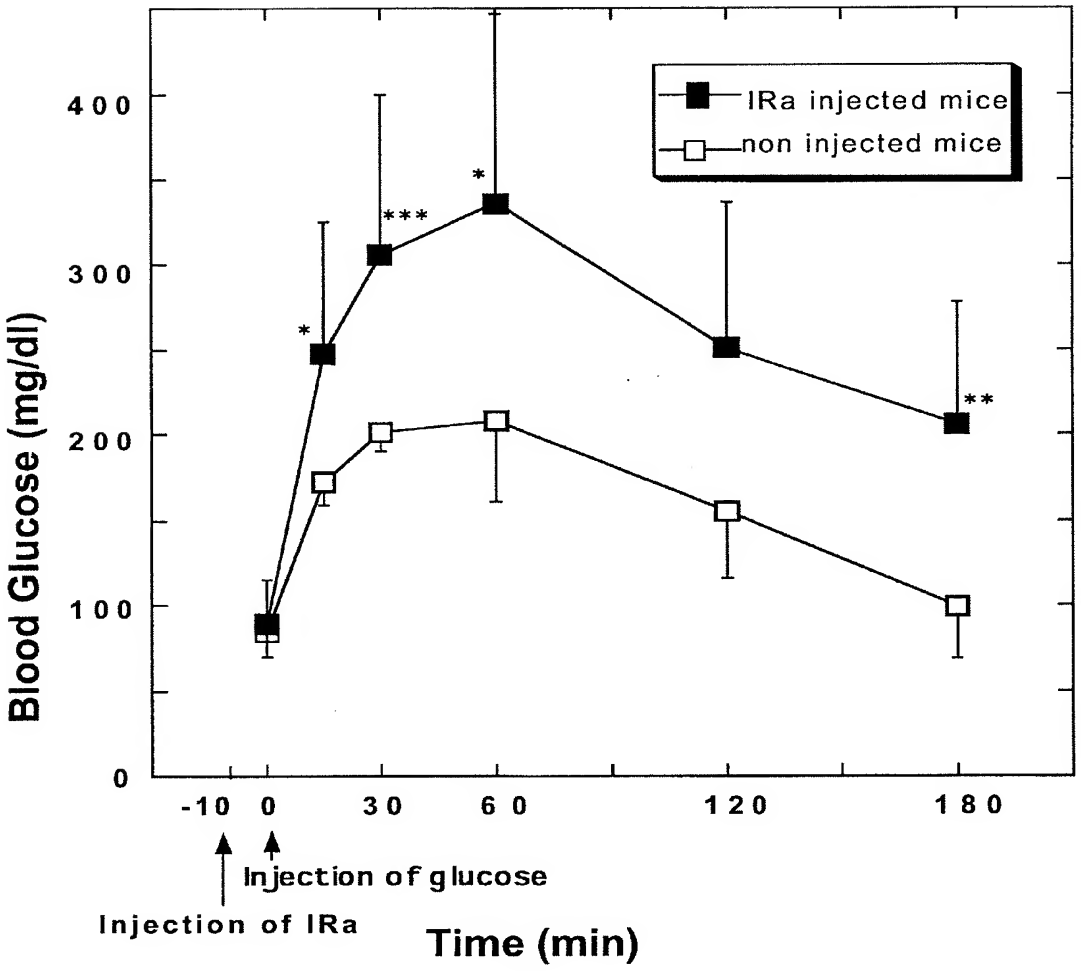
[Fig. 4]


3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD450
10
100
1000
10000
HIR-a(ng/mL)
OD450

[Fig. 5]


140
120
100
80
60
40
20
0
Blood Glucose (mg/dl)
*
* *
* *
* * *
* * *
* * *
IRa injected mice
non injected mice
-10 0
3 0
6 0
9 0
120
Injection of IRa
Time (min)

[Fig. 6]


IRa injected mice
non injected mice
Blood Glucose (mg/dl)
400
300
200
100
0
-10
0
30
60
120
180
*
***
*
**
Injection of glucose
Injection of IRa
Time (min)

[Document Name] Abstract

[Abstract]

[Problems to Be Solved]

To provide the methods for measuring free insulin receptor α-subunit in blood.

[Means for Solving the Problems]

Presence of free insulin receptor α-subunit in blood was discovered. Furthermore, methods for measuring the insulin receptor α-subunit was provided, the method comprising the steps of contacting the insulin receptor α-subunit in a blood sample with an antibody recognizing the insulin receptor α-subunit, and detecting the binding between the two. The free insulin receptor α-subunit in the blood inhibits the function of insulin and elevates blood glucose level. Therefore, methods for measuring free insulin receptor α-subunit in blood are useful for evaluating risk factors for diabetes.

[Selected Drawings] None